Asymmetries of reproductive isolation are reflected in directionalities of hybridization: Integrative evidence on the complexity of species boundaries

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Summary

- The complex nature of species boundaries has been a central topic in evolutionary biology ever since Darwin. Despite numerous separate studies on reproductive isolation and hybridization, their relationship remains under-investigated. Are the strengths and asymmetries of reproductive barriers reflected in the extent and directionalities of interspecific genetic exchange?
- We combined field, experimental, and molecular data to quantify strengths and asymmetries of sympatric reproductive barriers and hybridization between florally heteromorphic primroses. We also assessed whether generalist pollinators discriminate between different floral cues and contribute to reproductive isolation, a long-debated topic.
- Sympatric reproductive isolation is high but incomplete, and most phenotypic intermediates are genetic F1 hybrids, while backcrosses are rare, revealing low interspecific gene flow. Species integrity rests on multiple barriers, but ethological isolation is among the strongest, demonstrating that even generalist pollinators crucially contribute to the maintenance of species boundaries. Furthermore, reproductive barriers are weaker for *Primula veris* and short-styled plants, results corroborated by molecular data. Thus, in florally heteromorphic systems both species- and morph-dependent asymmetries affect permeability of species boundaries.
- Our study illustrates how the interactions between complex floral syndromes and pollinators shape species boundaries in unique, previously undescribed ways.

Keywords:
floral isolation
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INTRODUCTION

Hybridization and gene flow play pivotal roles in animal and plant evolution (Stebbins, 1950; Levin *et al.*, 1996; Arnold, 1997; Rieseberg & Carney, 1998; Mallet, 2005; Abbott *et al.*, 2013; Todesco *et al.*, 2016; Ostevik *et al.*, 2019). Natural hybridization occurs where divergent lineages meet, mate, and form at least some offspring of mixed ancestry (*i.e.,* hybrid zones; Harrison, 1993). The genotypic composition of hybrid zones inferred from molecular data provides valuable insights into the permeability of species boundaries at recent time scales, but cannot identify the type and strength of reproductive barriers that currently prevent or reduce genetic exchange (Dobzhansky, 1940; Mayr, 1940). To understand the complex nature of species boundaries at fine-grained resolution it is thus necessary to integrate population genetic analyses with field observations and experimental quantification of reproductive barriers, a rarely implemented approach (Moreira-Hernández & Muchhala, 2019).

Reproductive isolation is typically divided into premating and postmating stages (Coyne & Orr, 2004), which may both be modulated by mating/sexual system variation of the hybridizing species (Pickup *et al.*, 2019). Premating isolation consists of ecogeographic, phenological, mating system, and pollinator-mediated barriers, while postmating isolation consists of barriers preventing the formation, establishment, and reproduction of hybrids (Dobzhansky, 1940; Mayr, 1940; Grant, 1971). In mixed populations of animal-pollinated plant species, interspecific pollen flow can be restricted prior to mating by differences in flowering time, plant-pollinator interactions, or both (reviews by Campbell & Aldridge, 2006; Baack *et al.*, 2015). Differences in mating periods can form effective breeding barriers, as they limit the temporal window available for interspecific pollination (reviews by Baack *et al.*, 2015; Taylor & Friesen, 2017). Yet, numerous species co-flower, at least partially, thus having incomplete phenological barriers (Lowry *et al.*, 2008). Consequently, the realized transfer of pollen within *vs.* between co-flowering species depends largely on plant-pollinator interactions.

Pollinators are attracted or repelled by multiple floral cues (Gegear & Laverty, 2005; Nordström *et al.*, 2017). Interspecific differences in floral traits, including odour (Xu *et al.*, 2011), reward (Schemske & Bradshaw, 1999), colour (Schemske & Bradshaw, 1999; Chittka *et al.*, 2001; Bradshaw & Schemske, 2003), tube length (Anderson *et al.*, 2016; Minnaar *et al.*, 2019), and position of reproductive organs (Keller *et al.*, 2016) can contribute to floral isolation by altering the behaviour of pollinators and/or mechanically restricting interspecific pollen transfer.

Therefore, pollinator-mediated isolation has both behavioural and mechanical components (ethological and mechanical isolation; Grant, 1994), with the former often being more important than the latter, especially in insect-pollinated species lacking pollen packaging (Kay *et al.*, 2019).

It has been repeatedly demonstrated that pollen transfer is significantly reduced or prevented between species with contrasting pollinator types (Ramsey *et al.*, 2003; Xu *et al.*, 2011; Sun *et al.*, 2015). However, most plant species adopt generalist pollinators (Waser *et al.*, 1996) and the few available studies on plant-pollinator systems lacking highly specialized (*i.e.,* 1:1) plant-pollinator interactions have produced mixed results (Kephart & Theiss, 2003; Natalis & Wesselingh, 2012; Natalis & Wesselingh, 2013; Runquist *et al.*, 2014; Wang *et al.*, 2015; Tong & Huang, 2016; Kay *et al.*, 2019; Ma *et al.*, 2019). Moreover, premating barriers are often leaky (Kay & Sargent, 2009) and may be impermanent (Rosenblum *et al.*, 2012), hence postmating barriers are typically necessary to interrupt interspecific gene flow (Widmer *et al.*, 2008). These barriers include negative pollen-pistil interactions (Lewis & Crowe, 1958; Swanson *et al.*, 2004) and hybrid-seed inviability (Johnston *et al.*, 1980; Lester & Kang, 1998; Hämälä *et al.*, 2017). Finally, if hybrid seeds are formed and germinate, hybrid individuals may be outcompeted by parental individuals (Widmer *et al.*, 2008). Since reproductive barriers can occur at multiple stages, new integrative studies are needed to better understand both the effects of plant-pollinator interactions and the effects of postmating barriers on the maintenance of species boundaries.

Reproductive barriers can act asymmetrically, depending on which species or morph contributes the male and female gametes to hybrid formation. While species-dependent asymmetries have often been described in monomorphic species (Lowry *et al.*, 2008), morph-dependent asymmetries remain poorly known, probably because they can only be characterized in hermaphroditic species with heteromorphic individuals. Therefore, the study of such systems is required to tease apart species-dependent *vs*. morph-dependent asymmetries of reproductive isolation.

Despite the higher frequency of hybridization in plants than animals (Mallet, 2005), the number of genetically analysed plant hybrid zones is limited and information on the nature and strength of barriers to hybridization is wanting (Abbott, 2017). Furthermore, studies that integrate the experimental quantification of reproductive barriers with molecular analyses of gene flow are rare (Sambatti *et al.*, 2012), preventing a deeper understanding of the processes that shape species boundaries from current to recent time scales, respectively. Finally, the potential role of floral morphs in shaping reproductive barriers and hybridization remains under-investigated (Barrett,

2019), requiring in-depth analyses of hermaphroditic heteromorphic systems such as heterostylous primroses.

Heterostyly is a type of floral heteromorphism whereby two (distyly) or, more rarely, three (tristyly) genetically determined floral morphs differing in the reciprocal placement of reproductive organs (*i.e.,* reciprocal herkogamy; Ganders, 1979) occur in the same population. The two floral morphs of distylous species are referred to as long- and short-styled, hereafter L- and S-morph, respectively (Figs **1, S1**), also known as pins and thrums (Darwin, 1877). The spatial matching of reciprocal reproductive organs favours inter- over intra-morph pollen transfer (*i.e.,* disassortative pollination; Fig. **S1a)**, while a sporophytic incompatibility system reduces self- and intra-morph fertilization, thus promoting outcrossing (Wedderburn & Richards, 1990; Barrett, 2002). Heterostyly has been described in 119 genera across 28 angiosperm families (Lloyd & Webb, 1992; Barrett, 2002; Naiki, 2012; Barrett, 2019).

Ever since Darwin's seminal studies (Darwin, 1862; 1863; 1868; 1877), heterostyly has been most intensively investigated in *Primula* L. (primroses) *sensu* Mast *et al.* (Mast *et al.*, 2001; 2004; Mast & Conti, 2006; De Vos *et al.*, 2014), containing approximately 556 species, of which more than 82% are distylous. The heterostyly supergene of primroses has been recently characterized both functionally and molecularly as a hemizygous region present in S-morphs, but absent from L-morphs (Nowak *et al.*, 2015; Huu *et al.*, 2016; Li *et al.*, 2016; Burrows & McCubbin, 2017). Botanists and horticulturists alike have long known that hybridization frequently occurs between primrose species both in nature and under cultivation (Richards, 2003), and phylogenetic studies show that hybridization plays a pivotal role in the evolutionary history of *Primula* (Guggisberg *et al.*, 2008; 2009; Casazza *et al.*, 2012; Schmidt-Lebuhn *et al.*, 2012; Casazza *et al.*, 2013; Cianchi *et al.*, 2015; Boucher *et al.*, 2016). Reports of natural hybridization in *Primula* are usually based on morphological identification of hybrids (Kerner, 1875; Lotsy, 1925; Woodell, 1965; Kálmán *et al.*, 2004), but detailed population genetic analyses are scarce (but see Ma *et al.*, 2014; Xie *et al.*, 2017; Tendal *et al.*, 2018; Ma *et al.*, 2019).

Barriers to hybridization between *Primula* species have been studied for more than a century, but rarely in conjunction with molecular analyses of hybridization. Older studies, relying on manual pollination experiments, focused on postmating barriers, especially hybrid seed inviability (Darwin, 1877; Valentine, 1947; 1955). More recent studies investigated also premating isolation, including ethological (Wu & Zhang, 2010; Ma *et al.*, 2014; Xie *et al.*, 2017) and mechanical barriers, showing that floral isolation and hybrid seed inviability can be asymmetric

between species, morphs, or both (Keller *et al.*, 2016). Hence, in distylous primroses, species- and morph-dependent asymmetries may affect the permeability of species boundaries in complex, but poorly understood ways.

The hybridizing, heterostylous *Primula veris* L. and *P. vulgaris* Huds., two widespread species of *Primula* sect. *Primula* (Figs **1a, S1, S2**), represent an ideal system to gain an integrative understanding of hybridization and reproductive barriers because previous studies enable the framing of well-defined expectations to be tested with rigorous field, experimental, and molecular analyses. The two species do not form reciprocally monophyletic groups in molecular phylogenies of *Primula* sect. *Primula*, suggesting that introgression and/or incomplete lineage sorting played a role in their evolutionary history (Schmidt-Lebuhn *et al.*, 2012). Furthermore, previous hand-pollination experiments with British and Swedish accessions documented germination of hybrid seeds only in crosses with *P. veris* as mother, hence both post-mating barriers and hybridization are expected to be strongly asymmetric between species (Valentine, 1955). Additionally, patterns of floral variation in British and Hungarian contact sites prompted researchers to suggest that most phenotypically intermediate plants are F1 hybrids, while backcrosses are rare (Clifford, 1958; Mowat, 1961; Woodell, 1965; Kálmán *et al.*, 2004). However, the only available genetic study, using one nuclear (*ITS*), one plastid (*trnL*) and 10 microsatellite markers, supported introgression in Danish contact sites (Tendal *et al.*, 2018). Lastly, no genetic studies have examined whether floral morphs impose directionalities on hybridization, although morph-dependent asymmetries of reproductive barriers were shown in a different primrose species-pair (Keller *et al*., 2016).

Here, we combined field, experimental and molecular data to investigate whether the strengths and asymmetries of reproductive isolation are reflected in the directionalities of hybridization. Specifically, we tested the following hypotheses: (i) ethological isolation is the strongest barrier between *P. veris* and *P. vulgaris*; (ii) reproductive isolation is weaker for *P. veris* than *P. vulgaris* (asymmetric between species), hence *P. veris* is the more frequent maternal parent of F1 hybrids; (iii) distyly imposes morph-dependent asymmetries on both reproductive isolation and hybridization; and (iv) most phenotypically intermediate individuals are F1 hybrids, while backcrosses are rare, thus interspecific gene flow is scarce. By integrating our findings from different lines of evidence with previous results on the *P. veris*-*P. vulgaris* model system, the current study provides novel insights on the dynamic equilibrium between species divergence and merging.

MATERIAL AND METHODS

We performed field, experimental, and molecular analyses to assess the porosity of species boundaries in a natural contact site by quantifying the contribution of phenological, pollinator, and postmating barriers to sympatric isolation, estimating species- and morph-dependent asymmetries in reproductive barriers, characterizing floral cues that may be used by pollinators to discriminate among *P. veris*, *P. vulgaris*, and hybrids, and estimating gene flow and directionality of natural hybridization.

Study species

The early spring blooming *P. veris* and *P. vulgaris* are perennial, rosette-forming diploids (2n = 22) bearing distylous flowers on umbellate and rudimentary scapes, respectively (Richards, 2003). The generally similar flowers share yellow corollas with broad, v-notched lobes, but differ in other floral traits including shape, scent, and colour shade (Richards, 2003; Keller *et al.*, 2012), thus allowing for visual assignment of flowering individuals to *P. veris*, *P. vulgaris*, and phenotypic intermediates (*i.e.,* presumed hybrids) in the field (Figs **1a, S1b**). Both primulas are visited by at least 50 different insect species that have been divided into three distinct classes: large insects with long proboscises, small pollen-gathering bees, and very small insects that live inside the flower; the last category of insects is not considered as functional pollinators (Brys & Jacquemyn, 2009; Jacquemyn *et al.*, 2009). The main pollinators are bees, bumble bees, and bee flies (Brys & Jacquemyn, 2009; Jacquemyn *et al.*, 2009). The two species hybridize when they occur in close spatial proximity. The well documented local contact site of this study in the mountainous area above Montreux (VD, Switzerland; E 6°55’08; N 46°26’32’’) persisted for at least 162 years (Kerner, 1875; Lotsy, 1925), and is situated well within the large mosaic-like hybrid zone of the two species (Fig. **S2a**). For further information see **Methods S1 [Supporting Information].**

Reproductive isolation

To estimate the strength of sympatric isolation, we performed quantitative field surveys in the natural contact site and set up an experiment at the Botanical Garden of the University of Zürich, which is situated within a distance of ~2 km from natural populations of the two species and ~160 km from the studied contact site (Fig. **S2b**). Strengths of all reproductive isolation (*RI*) barriers were calculated following the method by Sobel and Chen (2014). Asymmetries of *RI* were

estimated following the method by Lowry *et al.* (2008) and their statistical significance tested using generalized linear mixed-effects model (GLMM) with contrasts, if applicable.

Field surveys were performed in six mixed patches of the natural contact site (Table **S1a**). On April 4th 2016, we exhaustively assigned anthetic (*i.e.,* with open flowers) long-styled (L-) and short-styled (S-) plants to either parental species or phenotypic hybrids; in 2018 we walked semi-permanent transects and weekly counted the number of flowers of anthetic L- and S-plants of phenotypic *P. veris*, *P. vulgaris*, and hybrids during the entire blooming periods of both species (Table **S1b-d**). Subsequently, we corrected the number of phenotypic hybrids in the contact site using the genetic analyses described below, demonstrating that 36 out of 46 phenotypic intermediates were genetic F1 hybrids, of which 35 had a *P. veris* mother and one a *P. vul*garis mother. The corrected frequencies of F1 hybrids at the contact site ($F1_{cor}$) were then applied in the following estimates of reproductive barriers: (i) phenological barriers between parents (RI_{phenoP}) and between $F1_{cor}$ and either parent (RI_{phenoH}), estimated from 2018 field-survey data comprising a total of 5193 visually identified plants (*P. veris*: 2727; *P. vulgaris*: 2129; hybrids: 337) and 25531 flowers (*P. veris*: 12747; *P. vulgaris*: 10088; hybrids: 2696); (ii) total sympatric isolation ($RI_{sympField}$), estimated from field data of both years.

For the experiment we used a design that closely resembled natural arrangements of plants in mixed patches of natural contact sites, where *P. veris* and *P. vulgaris* occur in areas containing individuals of either species, and areas that are intermixed (B. Keller, pers. obs.). Plants were randomly assigned to two treatments (Fig. **1b**): (i) intraspecific treatment, *i.e.* monospecific plots with either *P. veris* or *P. vulgaris* plants, with L- and S-morphs in alternating order and (ii) interspecific treatment, *i.e.,* heterospecific plots where both species were planted together in alternating order, with one plot consisting of short-styled *P. veris* and long-styled *P. vulgaris* and the other of long-styled *P. veris* and short-styled *P. vulgaris*. The two monospecific and the two heterospecific plots were arranged in a block that was replicated four times. The four blocks were at least 20 m apart, the four plots in a block 4 m apart, and individual plants within a plot 15 cm apart. To minimize pollen import from non-experimental plants, we removed all *Primula* plants within 20 meters of each block. Seeds produced in mono- and heterospecific plots should mainly result from intra- and interspecific cross pollinations, respectively, because (i) self- and intra-morph incompatibility is high (Wedderburn & Richards, 1990; Keller *et al.*, 2012); (ii) pollen carryover is strongly leptokurtic, with most of the pollen grains of *P. veris* transported within the first 6 m of the source plant and a long tail of pollen dispersal (Richards, 1997); and (iii) generalist

insects fly only short-distances between successive flower visits (Kevan & Baker, 1983), with more than 90% of all individuals of *Bombus sp.* flying less than 4 m between successive visits of *P. veris* flowers (Richards, 1997).

In experimental arrays, we observed pollinators' behaviour, recorded the number of openly pollinated flowers developing into a fruit (fruits), and counted, per fruit, the number of: (i) ovules developing into a seed (total seeds), (ii) full-sized dark brown seeds that most likely contained an embryo and/or a well-developed endosperm (filled seeds; see Valentine, 1955), and (iii) germinated seeds (*i.e.,* seedlings; Fig. **S1c**). We quantified ethological barriers between parental species from 36 hours and 40 minutes of pollinator observations. Such ethological barriers were subdivided into: (i) preference [*i.e.,* overexploitation of flowers of one species in the presence of the other species (Cock, 1978)], quantified by comparing the number of *P. veris* and *P. vulgaris* plants visited by a pollinator in a block (RI_{ethoP}); and (ii) constancy [*i.e.,* tendency of pollinators to move between flowers of one species by skipping flowers of the other species (Waser, 1986)], quantified by comparing the number of intra- *vs.* interspecific transitions in heterospecific plots (RI_{ethoC}). We calculated seed developmental ($RI_{seed\ dev}$) and germination barriers (RI_{germ}) from filled and germinated seeds, respectively. We estimated total premating isolation (RI_{pre}), total postmating isolation (RI_{post}), and total sympatric isolation ($RI_{sympExp}$) from individual barriers (*i.e.*, RI_{phenoP}, RI_{etho}, $RI_{seed\ dev}$, RI_{germ}, and RI_{phenoH}). For further details see **Methods S2 [Supporting Information].**

Differences of floral cues between *P. veris* and *P. vulgaris* and between hybrids and either parent

A crucial prerequisite for ethological isolation is that pollinators must be able to discriminate between species, which is only possible when their flowers differ in at least one, but usually multiple floral cues (Gegear & Laverty, 2005; Nordström *et al.*, 2017). Therefore, we quantified interspecific differences in floral colour and fragrance between *P. veris* and *P. vulgaris*, complementing previous knowledge on nectar-composition and floral morphological differences between the two species (Gardener & Gillman, 2001; Keller *et al.*, 2012; Abrahamczyk *et al.*, 2017). Floral scent and colour were measured on a total of 54 plants visually assigned to *P. veris* (20 plants), *P. vulgaris* (20 plants), and hybrids (14 plants) collected in five mixed patches at the Swiss contact site during peak flowering in spring 2018 (Table **S1e**). Spectral composition of

petals and chemical composition of floral bouquets were collected under standardized conditions in a greenhouse at the University of Zurich the day after plants were harvested. Display sizes (number of flowers per plant) were estimated from data collected in the 2018 field survey (see above; Table **S1b**). For further details see **Methods S3 [Supporting Information].**

Hybridization

To assess whether the strengths and directionalities of gene flow reflect those inferred from field and experimental quantification of reproductive isolation, we used recently published microsatellite loci (Triest *et al.*, 2015) and additionally developed nuclear PCR-based markers from previously generated RAD-Seq contigs of *P. veris* (Nowak *et al.*, 2015). The total number of nuclear markers used to genotype the contact site was 20: seven microsatellite and 13 RAD-based markers (Table **S2**). Additionally, we used the maternally inherited plastid *trnL* (Tendal *et al.*, 2018) to identify the maternal species of the hybrids and exon 3 of CYP^T to identify the maternal morph of the hybrids; CYP^T controls style length and is located in the hemizygous heterostyly supergene present only in S-morphs (Huu *et al.*, 2016; Li *et al.*, 2016). We also checked whether some of the generated *trnL* and CYP^T sequences could be assigned to *P. elatior* (whose sequences are available from GenBank and Huu et al. 2016, respectively), because this species is known to hybridize readily with *P. vulgaris* but rarely with *P. veris* (Jacquemyn *et al.*, 2009), and it is not reciprocally monophyletic to them (Schmidt-Lebuhn *et al.*, 2012). For further details see **Methods S4 [Supporting information].**

RESULTS

Reproductive isolation

Flowering periods of parents largely overlapped, hence RI_{phenoP} was modest in strength and symmetric between both species and morphs (Fig. **2a**, Table **1**). Blooming periods of hybrids overlapped extensively with the ones of both parents, thus RI_{phenoH} was weak and symmetric between both species and morphs.

We never witnessed a pollinator moving between plots during 36 hours and 40 minutes of pollinator observations on experimental arrays. Arrays were visited by 63 pollinators: 23 large bees (*Anthophora plumipes* Pallas, *Bombus humilis* Illiger*, B. hortorum* L., *B. terrestris* L. and *B. lucorum* L.), 32 bee flies (*Bombylius major* Latreille), and 8 small pollen-collecting bees that could not be assigned to species during experimental observations. Large bees and bee flies visited

heterospecific plots and monospecific plots of both species, while small bees visited only monospecific plots of *P. vulgaris* (Table **2a**). Large bees visited flowers of the two species randomly, while bee flies and small bees strongly favoured *P. vulgaris* over *P. veris*. Small bees cannot reach the sunken stigmas of S-morphs with their short proboscises, hence they can impose preference barriers (RI_{ethoP}) only on L-morphs (Table **3a**). Ethological isolation for preference across all pollinators (RI_{ethoP}) was thus stronger for exposed than sunken organs. For *P. veris*, large bees and bee flies performed fewer con- than heterospecific transitions. For *P. vulgaris*, large bees performed the same number of con- and heterospecific transitions and bee flies more con- than heterospecific transitions. Thus, RI_{ethoC}-values were negative for both pollinators in *P. veris*, while they were zero for large bees, but positive for bee flies in *P. vulgaris* (Tables **2b, 3b**). Hence, RI_{ethoC} was asymmetric between species for large bees, bee flies, and across all pollinators. Finally, total ethological isolation (RI_{etho}) was both stronger for *P. vulgaris* than *P. veris* and for L- than S-morphs (Table **1a**). Summarizing, RI_{etho} was highly asymmetric between species and moderately asymmetric between morphs of *P. veris* (Table **1b**).

Reproductive output was significantly lower in inter- than intraspecific treatments for both total and filled seeds, but not for fruits (Tables **4a-c, S3a-c**). The development of hybrid seeds was significantly lower for (i) *P. vulgaris* than *P. veris* at the stage *filled seeds* and (ii) L- than S-morphs at the stages *total seeds* and *filled seeds* (Table **4b,c**). Consequently, $RI_{seed\ dev}$, estimated from filled seeds, was stronger for *P. vulgaris* than *P. veris* and for L- than S-morphs (Table **1**). Finally, germination was significantly lower for hybrid than parental seeds and symmetric between both species and morphs (Tables **1, 4d, S3d**).

Total sympatric isolation estimated from individual barriers ($RI_{sympExp}$) was strong but incomplete (Table **1**). Phenological, hybrid seed development and/or germination were important barriers for both species, but ethological isolation was the strongest for *P. vulgaris*. Individual barriers were weaker for *P. veris* than *P. vulgaris* and for S- than L-morphs of *P. veris*, thus $RI_{sympExp}$ was weaker for *P. veris* and for S-plants of *P. veris*. In the natural contact site, frequencies of phenotypic *P. veris* and *P. vulgaris* ranged from 30.3% to 60.4% and from 33.6% to 61%, respectively, while frequencies of phenotypic hybrids ranged from 1.7% to 12.2% (Fig. **3**). Consequently, total sympatric isolation estimated from $F1_{cor}$ hybrid frequencies in each of the six mixed patches ($RI_{sympField}$) ranged from 0.47 to 0.945 for *P. veris* and from 0.985 to 0.998 for *P. vulgaris* (Table **S1d**). Thus, $RI_{sympField}$ was on average stronger for *P. vulgaris* than *P. veris*,

hence asymmetric between species (Table **1**). Summarizing, strengths and asymmetries of $RI_{sympExp}$ and $RI_{sympField}$ were congruent (Table **1**).

Differences of floral cues between *P. veris* and *P. vulgaris* and between hybrids and either parent

The quantifications of floral cues from the phenotypic assignment of plants to either pure or intermixed individuals were reliable because phenotypic and genetic assignments of plants to parental species coincided (Fig. **4a,c**): of the phenotypically intermediate plants, ~78% were F1 hybrids and ~20% were further genetically intermixed, while only one was genetically assigned to *P. vulgaris* (see below). Floral display sizes were similar in both parental species (contrast: $P = 0.512$), but significantly larger in phenotypic hybrids than in either parent (both contrasts: $P \leq 0.001$; GLMM: $F_{2,8207} = 96.031$; $P \leq 0.001$; Fig. **2b**). Petal colours differed significantly between *P. veris* and *P. vulgaris*, and were intermediate to both parents in hybrids (left panel in Fig. **2c**). Colour distances between parents (0.36 colour-hexagon units) and between phenotypic hybrids and either parent (hybrid *vs. P. veris* = 0.28; hybrid *vs. P. vulgaris* = 0.21) were all significantly larger than the threshold value of 0.1 colour-hexagon units for bees to show behavioural evidence for colour discrimination (right panel in Fig. **2c**; Chittka *et al.*, 2001).

We recorded a total of 42 scent compounds of floral (plant) origin (≤ 20 min retention time), of which 24 were reliably detected (Table **S4**). The total emitted scent was highest in *P. vulgaris* (262.730 ± 22.280 ng flower^{-1} hr^{-1}; mean ± standard error), lowest in *P. veris* (86.589 ± 10.509), and intermediate in phenotypic hybrids (113.604 ± 19.281); the difference was significant between *P. vulgaris* and each of the other two taxa (right panel Fig. **2d**). The floral bouquets were mainly composed of linalool (40%) for *P. veris*, nonanal (20%), benzaldehyde (20%) and β-pinene (19%) for *P. vulgaris*, and limonene (20%), β-pinene (20%), nonanal (17%), and benzaldehyde (16%) for the hybrids. For differences in the relative amounts of all compounds in the three taxa, see Fig. **S3** and Table **S4**. Floral scent profiles were significantly different among *P. veris*, *P. vulgaris*, and hybrids (ANOSIM: $R = 0.7089$; $P < 0.001$), highly distinct between parental species ($R = 0.9728$; $P < 0.001$), and more variable in phenotypic hybrids, but mostly intermediate between those of the two parents (Ve *vs.* Fx: $R = 0.4688$; $P < 0.001$; Vu *vs.* Fx: $R = 0.5145$; $P < 0.001$; left panel Fig. **2d**). Consequently, average scent dissimilarity was high between parents (83.7%), but lower between parents and phenotypic hybrids (Ve *vs.* Fx: 69.7%; Vu *vs.* Fx: 67.7%; Fig. **S4**).

Hybridization

i) Individual assignment to genotypic classes using nuclear markers

Assignments by STRUCTURE (threshold $Tq = 0.9$) and NewHybrids ($q > 0.9$) to parental species coincided with phenotypic assignments of individuals to *P. veris* and *P. vulgaris* with three exceptions: two of the 34 phenotypic *P. vulgaris* and one of the 32 phenotypic *P. veris* plants were in fact genetic backcrosses (Fig. **4a-c**). Thirty-six of the 46 phenotypic hybrids were genetic F1 hybrids, one a genetic *P. vulgaris*, two genetic backcrosses to *P. vulgaris*, one a genetic backcross to *P. veris*, while six plants could not be assigned with $q > 0.9$ to any of the six genotype classes. Several lines of evidence supported the predominance of F1 hybrids among genetically admixed individuals: (i) genetic differentiation indexes between hybrids and either parent were similarly low (pairwise F_{ST}: 0.303 *vs.* 0.251; pairwise G''_{ST}: 0.563 *vs.* 0.475), while parents were genetically highly differentiated from each other (pairwise F_{ST}: 0.738; pairwise G''_{ST}: 0.993; see Table **S5** for results of per locus values and Table **S6** for genetic diversity estimates); (ii) PCoA showed three distinct genetic clusters for *P. veris*, *P. vulgaris*, and F1 hybrids (Fig. **S5**); (iii) the discriminatory power of molecular markers was high for parents and simulated hybrids (Table **S7**).

ii) Identification of maternal species and morph using cpDNA and heterostyly supergene markers

Species of *Primula* section *Primula* differed at five and two polymorphic sites in *trnL* and exon 3 of CYP^T, respectively (Fig. **S6**). Polymorphisms in *trnL* suggested that all 31 plants genetically assigned to *P. veris* using the nuclear markers above ($q > 0.9$ in Fig. **4c**) had cpDNA from *P. veris* (Fig. **4d**), while 21 and 12 of the 33 plants genetically assigned to *P. vulgaris* had chloroplasts from *P. vulgaris* and *P. elatior*, respectively. Thirty-five of the plants genetically assigned to F1 hybrids using the nuclear markers above had cpDNA from *P. veris*, while one had cpDNA from *P. vulgaris*, mirroring *RI* species asymmetry in sympatry, weaker in *P. veris* than *P. vulgaris* (Table **1**). Polymorphisms at exon 3 of CYP^T suggested that all short-styled plants genetically assigned to *P. veris* and *P. vulgaris* using the nuclear markers above had CYP^T of their respective species (Fig. **4d**), while 11 and eight short-styled F1 hybrids had CYP^T from *P. veris* and *P. vulgaris*, respectively. Taken together, and assuming that self- and intra-morph incompatibility mechanisms are maintained across species boundaries (De Nettancourt, 2001; Keller *et al.*, 2016), the cpDNA and CYP^T results suggest that 11 F1 hybrids resulted from VE^{φ}_{S}-

$_{morph}$×VU♂$_{L\text{-}morph}$ crosses and eight from VE♀$_{L\text{-}morph}$×VU♂$_{S\text{-}morph}$ crosses. These genetic results mirrored *RI* morph asymmetry in sympatry, weaker in S- than L-morphs of *P. veris* (Table **1**).

DISCUSSION

The nature of species boundaries has long been a fundamental topic in evolutionary biology, yet whether the strengths and asymmetries of reproductive isolation are reflected in the extent and directionalities of hybridization remains largely unknown (Abbott, 2017). Here, we integrate results from field and experimental estimates of reproductive isolation with molecular analyses of genetic admixture to explain how species integrity is maintained from current to recent time scales, respectively.

Our field and experimental results corroborate key findings of previous studies, supporting the reliability of reproductive barrier estimates presented here. For example, observed pollinators and visitation frequencies were similar in experimental arrays (Table **2a**) and natural populations (Brys & Jacquemyn, 2009; Jacquemyn *et al.*, 2009; Deschepper *et al.*, 2018). Moreover, despite differences in numbers of pollinator visits to mono- and/or heterospecific plots (Table **2a**), numbers of total seeds per fruit from experimental arrays (Table **4b**) validated those from controlled crosses (Ernst, 1925; Valentine, 1955), indicating that observed variation in pollinator-visitation frequencies did not appreciably affect seed set. Furthermore, the stronger seed developmental barrier in *P. vulgaris* than *P. veris* inferred from experimental arrays (Table **4b**) corroborated both genetic data from the natural contact site (Fig. **4**) and prior results from manual crosses (Ernst, 1925; Valentine, 1955). Altogether, the internal consistency among our field, experimental and genetic results and external consistency with crucial elements of previous studies support the conclusion that our estimates of reproductive barriers are reliable.

High, but incomplete reproductive isolation between heterostylous primroses (Table **1**) matched the rarity of phenotypic intermediates in the natural contact site (Fig. **3**). Additionally, the fact that most phenotypic intermediates were genetic F1 hybrids, while backcrosses were rare (Fig. **4**), corroborates previous morphological surveys of contact sites in different localities (Clifford, 1958; Mowat, 1961; Woodell, 1965; Kálmán *et al.*, 2004), confirming low interspecific gene flow. Although species integrity rested on multiple barriers, the strongest one was ethological isolation (Table **1**), demonstrating that plant-pollinator interactions are important in maintaining species boundaries also in pollination systems lacking high levels of specialization, a long-debated topic (*e.g.,* Natalis & Wesselingh, 2013; Kay *et al.*, 2019).

Finally, reproductive barriers were weaker for *P. veris* than *P. vulgaris* and for S- than L-plants (Tables **1, 3**), implying that natural hybridization should be similarly asymmetric. Indeed, molecular analyses showed that most natural F1 hybrids had short-styled *P. veris* mothers (Fig. **4d**). In addition to widely reported species-dependent asymmetries of reproductive barriers in both monomorphic (Tiffin *et al.*, 2001; Lowry *et al.*, 2008) and heteromorphic systems (Valentine, 1955; Ma *et al.*, 2014; Keller *et al.*, 2016), morph-dependent asymmetries also affected sympatric isolation, corroborating previous findings in a different heterostylous species pair (Keller *et al.*, 2016). Our study is the first to demonstrate that the strengths and asymmetries of reproductive isolation between heterostylous species are reflected in the extent and directionalities of hybridization inferred from genetic data.

What restricts genetic exchange in hybrid zones?

Hybrid zones are 'natural laboratories' to test porosity of species boundaries (Hewitt, 1988). Prevalence of F1 hybrids and rarity of backcrosses among phenotypic intermediates in hybrid zones can be explained by strong parental isolation, selection against hybrids, infertility of F1 hybrids, and/or young age of contact sites (Milne *et al.*, 2003; Hersch-Green *et al.*, 2014). The studied contact site between *P. veris* and *P. vulgaris* has persisted for at least 162 years (Kerner, 1875; Lotsy, 1925) or approximately 81 generations, assuming a generation time of two years (Engler *et al.*, 2009), offering ample opportunities for hybridization and backcrossing. Hence, strong barriers to hybridization (Table **1**) and reduced fertility of F1 hybrids (Valentine, 1955; Kálmán *et al.*, 2003) best explain the observed lack of introgression (Fig. **4**), rather than age of contact site. Thus, results from both present and previous studies support the conclusion that strong isolating mechanisms maintain species integrity between geographically overlapping *P. veris* and *P. vulgaris*.

Multiple reproductive barriers are known to maintain species integrity in sympatry (Coyne & Orr, 2004; Lowry *et al.*, 2008; Baack *et al.*, 2015), but the role of ethological isolation in generalist-pollinated plants remains controversial (*e.g.,* Moreira-Hernández & Muchhala, 2019). Ethological isolation represented a major barrier between *P. veris* and *P. vulgaris,* indeed the strongest in the latter (Table **1**), although considerable barriers also occurred during flowering, seed development, and hybrid germination (Tables **1, 4**), corroborating previous studies (Melo *et al.*, 2014; Keller *et al.*, 2016; Peters & Weis, 2019). Thus, we demonstrate that pollinator-mediated barriers are crucial even in species with a putatively generalist pollination syndrome.

Plant-pollinator interactions shape ethological isolation in complex ways (*e.g.,* Borghi *et al.*, 2017). Pollinators' behavioural responses depend mainly on strength and divergence of floral signals (Chittka & Raine, 2006; Borghi *et al.*, 2017). Flowers of *P. veris* and *P. vulgaris*, while superficially similar (Fig. **1a**), differ significantly in floral morphology (Keller *et al.*, 2012), reward (Abrahamczyk *et al.*, 2017), and colour and scent, here quantified for the first time (Fig. **2c,d**). Interspecific floral differences were sufficiently large to alter pollinators' visitation patterns in absence (Table **2**) and presence of phenotypic intermediates (B. Keller, pers. obs.), imposing ethological isolation between the two parental species (Tables **1, 3**). Furthermore, pollinators use various (sub)sets of floral cues for long- *vs.* short-distance flower localization (Dötterl & Vereecken, 2010), adjusting foraging behaviour to frequency and spatial arrangement of co-flowering species (Natalis & Wesselingh, 2013). The two latter factors can differentially affect preference and constancy components of ethological isolation (Runquist *et al.*, 2014; Wang *et al.*, 2015): in primroses, preference was stronger than constancy (Table **3**). Moreover, contrasting pollinator types can contribute differently to ethological isolation due to distinct energy demands and preferences for floral cues (Waser & Ollerton, 2006; Natalis & Wesselingh, 2013). Small bees and bee flies favoured *P. vulgaris*, while large bees flew randomly between species, thus the former group contributed considerably more to reproductive isolation (Table **3**). Our results thus document the complex ways in which generalist pollinators shape reproductive barriers, providing novel evidence on a long-debated issue.

The long-held notion that postmating barriers do not vary significantly across geographic ranges (Coyne & Orr, 2004) has been recently challenged (Cutter, 2012; Corbett-Detig *et al.*, 2013). Results from the present and previous studies on different postmating barriers between *P. veris* and *P. vulgaris* clarify this debate. For example, seed development and germination barriers were considerably stronger in British/Swedish (Valentine, 1955) than Swiss contact sites (Table **1**). Phenotypic intermediates were more floriferous than either parent in the Swiss (Fig. **2b**) than in a Hungarian contact site (Kálmán *et al.*, 2003), implying stronger isolation in the latter at this postmating stage. Finally, a molecular analysis of a Danish contact site detected more introgression (Tendal *et al.*, 2018) than in the Swiss site (Fig. **4**), implying weaker postmating isolation in the former. By integrating evidence from the present study in Switzerland with previous studies in different geographic settings, it can be concluded that postmating isolation varies across distributional ranges, supporting the revised notion on geographic stability of postmating isolation.

Species- and morph-dependent asymmetries shape hybrid formation

The strength of sympatric isolation may be influenced by which species and morph serve as male or female parent in hybrid and backcross formation. While species-dependent asymmetries are known from both animals and plants (Tiffin *et al.*, 2001; Lowry *et al.*, 2008; Pickup *et al.*, 2019), morph-dependent asymmetries were recently discovered (Keller *et al.*, 2016), likely because they can only occur in hermaphroditic species with stable heteromorphisms, such as distylous primroses. Whether and how both types of asymmetries affect the direction of genetic exchange remains unknown.

Species-dependent asymmetries of pre-zygotic barriers differed from directionalities of gene flow in six of 10 species pairs (Moreira-Hernández & Muchhala, 2019), underscoring that total reproductive isolation, rather than pre- or post-zygotic barriers, should be estimated to predict directionality of introgression. In our study, species-dependent asymmetries of total reproductive isolation estimated from individual barriers and from genetically corrected field data were congruent (Table **1**). The most asymmetric barriers occurred during pollination and seed formation, with the former stronger than the latter. Specifically, pollinators were more faithful to *P. vulgaris* than *P. veris*, imposing a stronger barrier on the former, thus confirming that pollinator behaviour generates the most asymmetric premating barrier (Moreira-Hernández & Muchhala, 2019). Furthermore, reproductive isolation was equally asymmetric before and after mating between *P. veris* and *P. vulgaris* (Table **1**), contrary to previous findings that post-zygotic barriers were more asymmetric than pre-zygotic ones in a review of 19 species pairs (Lowry *et al.*, 2008). The fact that both premating and postmating barriers were stronger for *P. vulgaris* than *P. veris* predicts that most natural F1 hybrids should have *P. veris* mothers, as corroborated by genetic data (Fig. **4d**). By integrating experimental quantification of reproductive barriers, field surveys, and genetic analyses, our study for the first time shows that species-dependent asymmetries of reproductive isolation correctly predict the direction of natural hybridization.

The occurrence of different morphs in hermaphroditic species could affect reproductive barriers and natural hybridization in a morph-dependent manner. Between heterostylous species, reciprocity between high and low sexual organs can mechanically restrict pollen transfer between morphs in different ways. For example, interspecific pollen transfer operated by the long-tongued *Anthophora plumipes* is less efficient from high anthers of S-morph to high stigma of L-morph than *vice versa*, causing stronger isolation of the L-morph between *P. vulgaris* and *P. elatior*

(Keller *et al.*, 2016). Between *P. vulgaris* and *P. veris*, small bees with short tongues and bee flies with long tongues strongly preferred the former, whereas long-tongued large bees flew randomly between species (Table **2**). Additionally, small bees can only transfer pollen between high organs, whereas such long-tongued pollinators as large bees and bee flies can transfer pollen between both high and low organs (Deschepper *et al.*, 2018). The combined effects of species preference and mechanical constraints promoted intra- over interspecific pollen transfer, especially at high organ level, causing stronger reproductive isolation of L-morphs in both species (Table **3**). After mating, morph-dependent asymmetries occurred during hybrid seed development and germination, imposing especially strong isolation on *P. veris* L-morph (Tables **1, 4**) and confirming previous morph-asymmetry results between *P. vulgaris* and *P. elatior* (Keller et al. 2016). The congruent asymmetry of pre- and postmating isolation caused weaker total isolation for S- than L-plants of *P. veris*, implying that most F1 hybrids should be the product of compatible pollen transfer from *P. vulgaris* L-fathers to *P. veris* S-mothers. Our genetic results confirmed this prediction, for more F1 hybrids had both CYP^T and cpDNA from *P. veris* (Fig. **4d**). Our integrative analyses thus demonstrate for the first time that morph-dependent asymmetries of reproductive isolation predict the direction of hybridization in heteromorphic, hermaphroditic species.

Conclusions

By combining field, experimental, and genetic evidence we demonstrate that gene flow between *P. veris* and *P. vulgaris* is scarce and mainly restricted by barriers limiting pollen transfer and the formation of hybrid seedlings in both species, but less so for *P. veris* S-plants, which represented the main venue of hybridization (Table **1**; Fig. **4**). The same morph asymmetry of total sympatric reproductive isolation is now described in two hybridizing pairs of primroses (Table **1**; Fig. **4**; Keller *et al.*, 2016). Since floral morphs are genetically determined by the presence of the heterostyly supergene in S-morph and its absence from L-morphs (*e.g.,* Huu *et al.*, 2016), morph-specific effects directly or indirectly linked to the heterostyly supergene might contribute to modulating interspecific gene flow. Finally, recent simulation (Dagilis *et al.*, 2019) and empirical studies in other systems (Jordan *et al.*, 2018) suggested that even scarce gene flow might enable introgression of relatively small genomic regions that are advantageous for the recipient species (*i.e.,* adaptive introgression; Suarez-Gonzalez *et al.*, 2018). However, small portions of introgressed genomes cannot be detected with the genetic markers used here (McFarlane &

Pemberton, 2019), thus full genomic comparisons are underway to obtain fine-scale resolution of genetic exchange between heterostylous primroses.

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AUTHOR CONTRIBUTION

BK and EC conceived the idea and designed the project; BK and RG performed the study; BK, RG, EM-C, and MDN analysed the data; BK and EC wrote the paper with input from RG, EM-C, MDN, KK, and ST.

DATA AVAILABILITY STATEMENT

In Supporting Information, raw data are available for (i) the field survey (Table **S8**), (ii) the reproductive-isolation experiment (Table **S9**), (iii) the floral-colour quantification (Table **S10**), and (iv) the molecular analyses (Table **S11**).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Methods S1 Details of floral traits, functional pollinators, and habitat preferences for the two study species.
Methods S2 Quantification and analysis of strengths and asymmetries of reproductive isolation, **Methods S3** floral cues, and **Methods S4** hybridization in the studied contact site.

Figure S1: Heterostyly in *Primula*, hybridization between *Primula veris* and *P. vulgaris*, and sympatric reproductive barriers between *Primula veris* and *P. vulgaris* quantified with field and experimental data.

Figure S2: Map with distributional ranges of *P. veris* and *P. vulgaris* and their overlap in Europe and occurrence maps of both species in Switzerland.

Figure S3: Relative amounts of chemical compounds in the floral scents of parents and hybrids.

Figure S4: Pairwise dissimilarities of scent profiles between parents and hybrids.

Figure S5: Results of principal coordinate analysis (PCoA).

Figure S6: Alignment of previously published sequences of *trnL* and exon 3 of CYP^{T}.

Table S1: Details of the data collected in the studied contact site.

Table S2: Information of the RAD-based PCR markers.

Tables S3: GLMM results testing whether seed formation and germination differ among species, morphs, and intra- *vs.* interspecific treatments.

Table S4: Floral scent compositions and Kruskal-Wallis results testing whether individual compounds differ among *P. veris*, *P. vulgaris*, and hybrids.

Tables S5-S7: Genetic distance (**Table S5**), genetic diversity (**Table S6**), and discriminatory power of used molecular markers (**Table S7**).

Tables S8-10: Raw data of field survey (**Table S8**), reproductive-isolation experiment (**Table S9**) and floral-colour quantification (**Table S10**).

Table S11: Sequence polymorphisms of RAD-based loci, *trnL* and exon 3 of *CYP*^{T}, and allele sizes of microsatellite loci.

REFERENCES

Abbott RJ. 2017. Plant speciation across environmental gradients and the occurrence and nature of hybrid zones. *Journal of Systematics and Evolution* **55**(4): 238-258.

Abbott RJ, Albach D, Ansell S, Arntzen JW, Baird SJE, Bierne N, Boughman J, Brelsford A, Buerkle CA, Buggs R, et al. 2013. Hybridization and speciation. *Journal of Evolutionary Biology* **26**(2): 229-246.

Abrahamczyk S, Kessler M, Hanley D, Karger DN, Muller MP, Knauer AC, Keller F, Schwerdtfeger M, Humphreys AM. 2017. Pollinator adaptation and the evolution of floral nectar sugar composition. *Journal of Evolutionary Biology* **30**(1): 112-127.

Anderson B, Pauw A, Cole WW, Barrett SCH. 2016. Pollination, mating and reproductive fitness in a plant population with bimodal floral-tube length. *Journal of Evolutionary Biology* **29**(8): 1631-1642.

Anderson EC, Thompson EA. 2002. A model-based method for identifying species hybrids using multilocus genetic data. *Genetics* **160**(3): 1217-1229.

Arnold ML. 1997. *Natural hybridization and evolution*. New York, Oxford: Oxford University Press.

Baack E, Melo MC, Rieseberg LH, Ortiz-Barrientos D. 2015. The origins of reproductive isolation in plants. *New Phytologist* **207**(4): 968-984.

Barrett SCH. 2002. The evolution of plant sexual diversity. *Nature Reviews Genetics* **3**(4): 274-284.

Barrett SCH. 2019. 'A most complex marriage arrangement': recent advances on heterostyly and unresolved questions. *New Phytologist* **224**(3): 1051-1067.

Borghi M, Fernie AR, Schiestl FP, Bouwmeester HJ. 2017. The sexual advantage of looking, smelling, and tasting good: the metabolic network that produces signals for pollinators. *Trends in Plant Science* **22**(4): 338-350.

Boucher FC, Casazza G, Szövényi P, Conti E. 2016. Sequence capture using RAD probes clarifies phylogenetic relationships and species boundaries in *Primula* sect. *Auricula*. *Molecular Phylogenetics and Evolution* **104**: 60-72.

Bradshaw HD, Schemske DW. 2003. Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. *Nature* **426**(6963): 176-178.

Brys R, Jacquemyn H. 2009. Biological Flora of the British Isles: *Primula veris* L. *Journal of Ecology* **97**(3): 581-600.

Burrows BA, McCubbin AG. 2017. Sequencing the genomic regions flanking S-linked PvGLO sequences confirms the presence of two GLO loci, one of which lies adjacent to the style-length determinant gene CYP734A50. *Plant Reproduction* **30**(1): 53-67.

Campbell DR, Aldridge G 2006. Floral biology of hybrid zones. In: Harder LD, Barrett SCH eds. *Ecology and evolution of flowers*. Oxford, UK: Oxford University Press, 326-345.

Casazza G, Granato L, Minuto L, Conti E. 2012. Polyploid evolution and Pleistocene glacial cycles: A case study from the alpine primrose *Primula marginata* (Primulaceae). *BMC Evolutionary Biology* **12**(1): 56.

Casazza G, Grassi F, Zecca G, Mariotti MG, Guerrina M, Minuto L. 2013. Phylogeography of *Primula allionii* (Primulaceae), a narrow endemic of the Maritime Alps. *Botanical Journal of the Linnean Society* **173**(4): 637-653.

Chittka L, Raine NE. 2006. Recognition of flowers by pollinators. *Current Opinion in Plant Biology* **9**(4): 428-435.

Chittka L, Spaethe J, Schmidt A, Hickelsberger A 2001. Adaptation, constraint, and chance in the evolution of flower color and pollinator color vision. In: Chittka L, Thomson JD eds. *Cognitive ecology of pollination - animal behaviour and floral evolution*. New York, USA: Cambridge University Press, 106-126.

Cianchi R, Arduino P, Mosco MC, Bullini L. 2015. Evidence of hybrid speciation in the North American primroses *Primula suffrutescens, P. parryi, P. rusbyi,* and *P. angustifolia* (Primulaceae). *Plant Biosystems* **149**(2): 229-234.

Clifford HT. 1958. Studies in British Primulas. VI. On introgression between primrose (*Primula vulgaris* Huds.) and cowslip (*P. veris* L.). *New Phytologist* **57**(1): 1-10.

Cock MJW. 1978. The assessment of preference. *Journal of Animal Ecology* **47**(3): 805-816.

Corbett-Detig RB, Zhou J, Clark AG, Hartl DL, Ayroles JF. 2013. Genetic incompatibilities are widespread within species. *Nature* **504**(7478): 135-137.

Coyne JA, Orr HA. 2004. *Speciation*. Sunderland, Massachusetts (USA): Sinauer Associates, Inc.

Cutter AD. 2012. The polymorphic prelude to Bateson–Dobzhansky–Muller incompatibilities. *Trends in Ecology & Evolution* **27**(4): 209-218.

Dagilis AJ, Kirkpatrick M, Bolnick DI. 2019. The evolution of hybrid fitness during speciation. *PLoS Genet* **15**(5): e1008125.

Darwin C. 1862. On the two forms, or dimorphic condition, in the species of *Primula*, and on their remarkable sexual relations. *Botanical Journal of the Linnean Society* **6**: 77-96.

Darwin C. 1863. On the existence of two forms, and on their reciprocal sexual relation, in several species of the genus Linum. *Journal of the Proceedings of the Linnean Society of London. Botany* **7**(26): 69-83.

Darwin C. 1868. On the specific difference between *Primula veris*, Brit. Fl. (var *officinalis* of Linn.), *P. vulgaris*, Brit. Fl. (var *acaulis* Linn.), and *P. elatior*, Jacq.; and on the hybrid nature of the common oxlip. With supplementary remarks on naturally-produced hybrids in the genus *Verbascum*. *Botanical Journal of the Linnean Society* **10**: 437-454.

Darwin C. 1877. *The different forms of flowers on plants of the same species*. London, UK: John Murray.

De Nettancourt D. 2001. *Incompatibility and incongruity in wild and cultivated plants*. Berlin, Germany: Springer.

De Vos JM, Hughes CE, Schneeweiss GM, Moore BR, Conti E. 2014. Heterostyly accelerates diversification *via* reduced extinction in primroses. *Proceedings of the Royal Society B: Biological Sciences* **281**(1784): 20140075.

Deschepper P, Brys R, Jacquemyn H. 2018. The impact of flower morphology and pollinator community composition on pollen transfer in the distylous *Primula veris*. *Botanical Journal of the Linnean Society* **186**(3): 414-424.

Dobzhansky T. 1940. Speciation as a stage in evolutionary divergence. *American Naturalist* **74**: 312-321.

Dötterl S, Vereecken NJ. 2010. The chemical ecology and evolution of bee–flower interactions: a review and perspectives. *Canadian Journal of Zoology* **88**(7): 668-697.

Dyer AG, Chittka L. 2004. Biological significance of distinguishing between similar colours in spectrally variable illumination: bumblebees (*Bombus terrestris*) as a case study. *Journal of Comparative Physiology a-Neuroethology Sensory Neural and Behavioral Physiology* **190**(2): 105-114.

Engler R, Randin CF, Vittoz P, Czáka T, Beniston M, Zimmermann NE, Guisan A. 2009. Predicting future distributions of mountain plants under climate change: does dispersal capacity matter? *Ecography* **32**(1): 34-45.

Ernst A. 1925. Zur Kenntnis des Artbastardes *Primula variabilis* Goupil (*Pr. vulgaris* x *veris*) und seiner Nachkommenschaft. *Zeitschrift für induktive Abstammungs- und Verbungslehre* **27**: 233-235.

Ganders FR. 1979. The biology of heterostyly. *New Zealand Journal of Botany* **17**(4): 607-635.

Gardener MC, Gillman MP. 2001. Analyzing variability in nectar amino acids: composition is less variable than concentration. *Journal of Chemical Ecology* **27**(12): 2545-2558.

Gegear RJ, Laverty TM. 2005. Flower constancy in bumblebees: a test of the trait variability hypothesis. *Animal Behaviour* **69**(4): 939-949.

Grant V. 1971. *Plant speciation*. New York, USA: Columbia University Press.

Grant V. 1994. Modes and origins of mechanical and ethological isolation in angiosperms. *Proceedings of the National Academy of Sciences* **91**(1): 3-10.

Guggisberg A, Baroux C, Grossniklaus U, Conti E. 2008. Genomic origin and organization of the allopolyploid *Primula egaliksensis* investigated by *in situ* hybridization. *Annals of Botany* **101**: 919-927.

Guggisberg A, Mansion G, Conti E. 2009. Disentangling reticulate evolution in an arctic-alpine polyploid complex. *Systematic Biology* **58**(1): 55-73.

Hämälä T, Mattila TM, Leinonen PH, Kuittinen H, Savolainen O. 2017. Role of seed germination in adaptation and reproductive isolation in *Arabidopsis lyrata*. *Molecular Ecology* **26**(13): 3484-3496.

Harrison RG. 1993. *Hybrid zones and the evolutionary process*. New York, USA: Oxford University Press.

Hersch-Green E, Allan G, Whitham T. 2014. Genetic analysis of admixture and patterns of introgression in foundation cottonwood trees (Salicaceae) in southwestern Colorado, USA. *Tree Genetics & Genomes* **10**(3): 527-539.

Hewitt GM. 1988. Hybrid zones - natural laboratories for evolutionary studies. *Trends in Ecology & Evolution* **3**(7): 158-167.

Huu CN, Kappel C, Keller B, Sicard A, Takabayashi Y, Breuninger H, Nowak MD, Bäuerle I, Himmelbach A, Burkart M, et al. 2016. Presence versus absence of CYP734A50 underlies the style-length dimorphism in primroses. *Elife* **5**: e17956.

Jacquemyn H, Endels P, Brys R, Hermy M, Woodell SRJ. 2009. Biological Flora of the British Isles: *Primula vulgaris* Huds. (*P. acaulis* (L.) Hill). *Journal of Ecology* **97**(4): 812-833.

Johnston SA, den Nijs TPM, Peloquin SJ, Hanneman RE, Jr. 1980. The significance of genic balance to endosperm development in interspecific crosses. *Theoretical and Applied Genetics* **57**(1): 5-9.

Jordan CY, Lohse K, Turner F, Thomson M, Gharbi K, Ennos RA. 2018. Maintaining their genetic distance: Little evidence for introgression between widely hybridizing species of *Geum* with contrasting mating systems. *Molecular Ecology* **27**(5): 1214-1228.

Kálmán K, Medvegy A, Mihalik E. 2003. Characteristics of infructescence and seed yield in a *Primula* hybrid zone. *Acta Botanica Hungarica* **45**(3-4): 323-338.

Kálmán K, Medvegy A, Mihalik E. 2004. Pattern of the floral variation in the hybrid zone of two distylous *Primula* species. *Flora* **199**(3): 218-227.

Kay KM, Sargent RD. 2009. The role of animal pollination in plant speciation: integrating ecology, geography, and genetics. *Annual Review of Ecology Evolution and Systematics* **40**: 637-656.

Kay KM, Zepeda AM, Raguso RA. 2019. Experimental sympatry reveals geographic variation in floral isolation by hawkmoths. *Annals of Botany* **123**(2): 405–413.

Keller B, de Vos JM, Conti E. 2012. Decrease of sexual organ reciprocity between heterostylous primrose species, with possible functional and evolutionary implications. *Annals of Botany* **110**(6): 1233-1244.

Keller B, de Vos JM, Schmidt-Lebuhn AN, Thomson JD, Conti E. 2016. Both morph- and species-dependent asymmetries affect reproductive barriers between heterostylous species. *Ecology and Evolution* **6**(17): 6223-6244.

Kephart S, Theiss K. 2003. Pollinator-mediated isolation in sympatric milkweeds (*Asclepias*): do floral morphology and insect behavior influence species boundaries? *New Phytologist* **161**(1): 265-277.

Kerner A. 1875. Die Primulaceen-Bastarte der Alpen. *Oesterreichische Botanische Zeitschrift* **3**: 77-82.

Kevan PG, Baker HG. 1983. Insects as Flower Visitors and Pollinators. *Annual Review of Entomology* **28**(1): 407-453.

Lester RN, Kang JH. 1998. Embryo and endosperm function and failure in *Solanum species* and hybrids. *Annals of Botany* **82**(4): 445-453.

Levin DA, Francisco-Ortega J, Jansen RK. 1996. Hybridization and the extinction of rare plant species. *Conservation Biology* **10**(1): 10-16.

Lewis D, Crowe LK. 1958. Unilateral interspecific incompatibility in flowering plants. *Heredity* **12**(2): 233-256.

Li J, Cocker JM, Wright J, Webster MA, McMullan M, Dyer S, Swarbreck D, Caccamo M, van Oosterhout C, Gilmartin PM. 2016. Genetic architecture and evolution of the S locus supergene in *Primula vulgaris*. *Nature Plants* **2**: 16188.

Lloyd DG, Webb CJ 1992. The evolution of heterostyly. In: Barrett SCH ed. *Evolution and function of heterostyly*. Berlin, Germany: Springer, 151-178.

Lotsy JP. 1925. Studien an wilden Bastarden zwischen verschiedenen Linneonten. *Genetica* **V7**(3): 177-233.

Lowry DB, Modliszewski JL, Wright KM, Wu CA, Willis JH. 2008. The strength and genetic basis of reproductive isolating barriers in flowering plants. *Philosophical Transactions of the Royal Society B: Biological Sciences* **363**(1506): 3009-3021.

Ma Y, Marczewski T, Xue D, Wu Z, Liao R, Sun W, Marczewski J. 2019. Conservation implications of asymmetric introgression and reproductive barriers in a rare primrose species. *BMC Plant Biology* **19**(1): 286.

Ma YP, Xie WJ, Tian XL, Sun WB, Wu ZK, Milne R. 2014. Unidirectional hybridization and reproductive barriers between two heterostylous primrose species in north-west Yunnan, China. *Annals of Botany* **113**(5): 763-775.

Mallet J. 2005. Hybridization as an invasion of the genome. *Trends in Ecology & Evolution* **20**(5): 229-237.

Mast AR, Conti E. 2006. The primrose path to heterostyly. *New Phytologist* **171**(3): 439-442.

Mast AR, Feller DMS, Kelso S, Conti E. 2004. Buzz-pollinated *Dodecatheon* originated from within the heterostylous *Primula* subgenus *Auriculastrum* (Primulaceae): A seven-region

cpDNA phylogeny and its implications for floral evolution. *American Journal of Botany* **91**(6): 926-942.

Mast AR, Kelso S, Richards AJ, Lang DJ, Feller DMS, Conti E. 2001. Phylogenetic relationships in *Primula* L. and related genera (Primulaceae) based on noncoding chloroplast DNA. *International Journal of Plant Sciences* **162**(6): 1381-1400.

Mayr E. 1940. Speciation phenomena in birds. *The American Naturalist* **74**(752): 249-278.

McFarlane SE, Pemberton JM. 2019. Detecting the true extent of introgression during anthropogenic hybridization. *Trends in Ecology & Evolution* **34**(4): 315-326.

Melo MC, Grealy A, Brittain B, Walter GM, Ortiz-Barrientos D. 2014. Strong extrinsic reproductive isolation between parapatric populations of an Australian groundsel. *New Phytologist* **203**(1): 323-334.

Milne RI, Terzioglu S, Abbott RJ. 2003. A hybrid zone dominated by fertile F_1s: maintenance of species barriers in *Rhododendron*. *Molecular Ecology* **12**(10): 2719-2729.

Minnaar C, de Jager ML, Anderson B. 2019. Intraspecific divergence in floral-tube length promotes asymmetric pollen movement and reproductive isolation. *New Phytologist* **224**(3): 1160-1170.

Moreira-Hernández JI, Muchhala N. 2019. Importance of pollinator-mediated interspecific pollen transfer for Angiosperm evolution. *Annual Review of Ecology, Evolution, and Systematics* **50**(1): 191-217.

Mowat AB. 1961. An investigation of mixed populations of *Primula veris* and *P. vulgaris*. *Transactions of the Botanical Society of Edinburgh* **39**(2): 206-211.

Naiki A. 2012. Heterostyly and the possibility of its breakdown by polyploidization. *Plant Species Biology* **27**(1): 3-29.

Natalis LC, Wesselingh RA. 2012. Shared pollinators and pollen transfer dynamics in two hybridizing species, *Rhinanthus minor* and *R. angustifolius*. *Oecologia* **170**(3): 709-721.

Natalis LC, Wesselingh RA. 2013. Parental frequencies and spatial configuration shape bumblebee behavior and floral isolation in hybridizing *Rhinanthus*. *Evolution* **67**(6): 1692-1705.

Nordström K, Dahlbom J, Pragadheesh VS, Ghosh S, Olsson A, Dyakova O, Suresh SK, Olsson SB. 2017. In situ modeling of multimodal floral cues attracting wild pollinators across environments. *Proceedings of the National Academy of Sciences* **114**(50): 13218–13223.

Nowak MD, Russo G, Schlapbach R, Cuong NH, Lenhard M, Conti E. 2015. The draft genome of *Primula veris* yields insights into the molecular basis of heterostyly. *Genome Biology* **16**(1): 12.

Ostevik KL, Rifkin JL, Xia H, Rausher MD. 2019. Asymmetric gene flow causes cascading reproductive isolation. *bioRxiv*: 767970.

Peters MAE, Weis AE. 2019. Isolation by phenology synergizes isolation by distance across a continuous landscape. *New Phytologist* **224**(3): 1215-1228.

Pickup M, Brandvain Y, Fraisse C, Yakimowski S, Barton NH, Dixit T, Lexer C, Cereghetti E, Field DL. 2019. Mating system variation in hybrid zones: facilitation, barriers and asymmetries to gene flow. *New Phytologist* **224**: 1035–1047.

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics* **155**(2): 945-959.

Ramsey J, Bradshaw HD, Schemske DW. 2003. Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). *Evolution* **57**(7): 1520-1534.

Richards AJ. 1997. *Plant breeding systems*. London, UK: Chapmann and Hall.

Richards AJ. 2003. *Primula*. Portland, OR, USA: Timber Press.

Rieseberg LH, Carney SE. 1998. Plant hybridization. *New Phytologist* **140**(4): 599-624.

Rosenblum EB, Sarver BAJ, Brown JW, Des Roches S, Hardwick KM, Hether TD, Eastman JM, Pennell MW, Harmon LJ. 2012. Goldilocks Meets Santa Rosalia: An ephemeral speciation model explains patterns of diversification across time scales. *Evolutionary Biology* **39**(2): 255-261.

Runquist RDB, Chu E, Iverson JL, Kopp JC, Moeller DA. 2014. Rapid evolution of reproductive isolation between incipient outcrossing and selfing *Clarkia* species. *Evolution* **68**(10): 2885-2900.

Sambatti JBM, Strasburg JL, Ortiz-Barrientos D, Baack EJ, Rieseberg LH. 2012. Reconciling extremely strong barriers with high levels of gene exchange in annual sunflowers. *Evolution* **66**(5): 1459-1473.

Schemske DW, Bradshaw HD, Jr. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proceedings of the National Academy of Sciences, USA* **96**(21): 11910-11915.

Schmidt-Lebuhn AN, Keller B, De Vos JM, Conti E. 2012. Phylogenetic analysis of *Primula* section *Primula* reveals rampant non-monophyly among morphologically distinct species. *Molecular Phylogenetics and Evolution* **65**(1): 23-34.

Sobel JM, Chen GF. 2014. Unification of methods for estimating the strength of reproductive isolation. *Evolution* **68**(5): 1511-1522.

Stebbins GL. 1950. *Variation and evolution in plants*. New York, USA: Columbia University Press.

Suarez-Gonzalez A, Lexer C, Cronk QCB. 2018. Adaptive introgression: a plant perspective. *Biology Letters* **14**(3): 20170688.

Sun M, Schlüter PM, Gross K, Schiestl FP. 2015. Floral isolation is the major reproductive barrier between a pair of rewarding orchid sister species. *Journal of Evolutionary Biology* **28**(1): 117-129.

Swanson R, Edlund AF, Preuss D. 2004. Species specificity in pollen-pistil interactions. *Annual Review of Genetics* **38**(1): 793-818.

Taylor RS, Friesen VL. 2017. The role of allochrony in speciation. *Molecular Ecology* **26**(13): 3330-3342.

Tendal K, Ørgaard M, Larsen B, Pedersen C. 2018. Recurrent hybridisation events between *Primula vulgaris*, *P. veris* and *P. elatior* (Primulaceae, Ericales) challenge the species boundaries: Using molecular markers to re-evaluate morphological identifications. *Nordic Journal of Botany* **36**(9): e01778.

Tiffin P, Olson MS, Moyle LC. 2001. Asymmetrical crossing barriers in angiosperms. *Proceedings of the Royal Society B: Biological Sciences* **268**(1469): 861-867.

Todesco M, Pascual MA, Owens GL, Ostevik KL, Moyers BT, Hubner S, Heredia SM, Hahn MA, Caseys C, Bock DG, et al. 2016. Hybridization and extinction. *Evolutionary Applications* **9**(7): 892-908.

Tong Z-Y, Huang S-Q. 2016. Pre- and post-pollination interaction between six co-flowering *Pedicularis* species via heterospecific pollen transfer. *New Phytologist* **211**(4): 1452-1461.

Triest L, Sierens T, Van Rossum F. 2015. Multiplexing 15 microsatellite loci for European primrose (*Primula vulgaris*). *Conservation Genetics Resources* **7**(1): 279-281.

Valentine DH. 1947. Studies in British Primulas. I. Hybridization between primrose and oxlip (*Primula vulgaris* Huds. and *P. elatior* Schreb.). *New Phytologist* **46**(2): 229-253.

Valentine DH. 1955. Studies in British Primulas. IV. Hybridization between *Primula vulgaris* Huds. and *P. veris* L. *New Phytologist* **54**(1): 70-80.

Wang L-L, Zhang C, Tian B, Sun X-D, Guo W, Zhang T-F, Yang Y-P, Duan Y-W. 2015. Reproductive isolation is mediated by pollen incompatibility in sympatric populations of two *Arnebia* species. *Ecology and Evolution* **5**(24): 5838-5846.

Waser NM. 1986. Flower constancy: Definition, cause, and measurement. *American Naturalist* **127**(5): 593-603.

Waser NM, Chittka L, Price MV, Williams NM, Ollerton J. 1996. Generalization in pollination systems, and why it matters. *Ecology* **77**(4): 1043-1060.

Waser NM, Ollerton J. 2006. *Plant-pollinator interactions from specialization to generalization*. Chicago, USA: University of Chicago Press.

Wedderburn FM, Richards AJ. 1990. Variation in within-morph incompatibility inhibition sites in heteromorphic *Primula* L. *New Phytologist* **116**(1): 149-162.

Widmer A, Lexer C, Cozzolino S. 2008. Evolution of reproductive isolation in plants. *Heredity* **102**(1): 31–38.

Woodell SRJ. 1965. Natural hybridization between the cowslip (*Primula veris* L.) and the primrose (*P. vulgaris* Huds.) in Britain. *Watsonia* **6**(3): 190-202.

Wu ZK, Zhang CQ. 2010. Comparative study of pollination biology of two closely related alpine *Primula* species, namely *Primula beesiana* and *P. bulleyana* (Primulaceae). *Journal of Systematics and Evolution* **48**(2): 109-117.

Xie Y, Zhu X, Ma Y, Zhao J, Li L, Li Q. 2017. Natural hybridization and reproductive isolation between two *Primula* species. *Journal of Integrative Plant Biology* **59**(8): 526-530.

Xu S, Schlüter PM, Scopece G, Breitkopf H, Gross K, Cozzolino S, Schiestl FP. 2011. Floral isolation the main reproductive barrier among closely related sexual deceptive orchids. *Evolution* **65**(9): 2606-2620.

Table 1: Mean strengths of sympatric reproductive barriers (*RI*) with standard errors (a) and asymmetries (AS) between morphs and species (b) for long-styled (L-) and short-styled (S-) morphs of *Primula veris* (VE) and *P. vulgaris* (VU)

				Premating barriers			Postmating barriers				Total sympatric isolation		
		Stages in life cycle:		Flowering	Pollen transfer	Total	Hybrid seed formation	Hybrid seed germination	Hybrid flowering	Total			
		Species	Morph	RI_{phenoP}	RI_{etho}	RI_{pre}	$RI_{seed\ dev}$	RI_{germ}	RI_{phenoH}	RI_{post}	$RI_{sympExp}$	$RI_{sympField}$[1] 2016	$RI_{sympField}$[1] 2018
(a)	Barrier strength	VE	L	0.230±0.097	0.374 —	0.628 —	0.159±0.058	0.344±0.037	0.123±0.021	0.567 —	0.881 —	0.863±0.073	0.753±0.168
	(mean ± standard error)		S	0.258±0.087	0.180 —	0.535 —	0.049±0.089	0.245±0.408	0.098±0.052	0.378—	0.759 —	0.863±0.073	0.753±0.168
		VU	L	0.266±0.100	0.782 —	0.863 —	0.376±0.173	0.399±0.310	0.197±0.049	0.769 —	0.981 —	0.996±0.002	0.993±0.005
			S	0.314±0.094	0.686 —	0.825 —	0.257±0.211	0.340±0.430	0.170±0.030	0.658 —	0.961 —	0.996±0.002	0.993±0.005
(b)	AS between morphs	VE	—	0.028	0.193	0.093	0.110	0.100	0.025	0.189	0.122	—	—
		VU	—	0.047	0.096	0.038	0.119	0.059	0.028	0.111	0.020	—	—
	AS between species	—	L	0.037	0.409	0.235	0.216	0.054	0.075	0.202	0.100	0.133	0.240
		—	S	0.056	0.506	0.290	0.208	0.095	0.072	0.280	0.202	0.133	0.240

Phenological reproductive isolation (*RI*) of parents (RI_{phenoP}), total ethological isolation across all pollinators (RI_{etho}), seed developmental isolation ($RI_{seed\ dev}$), hybrid seed germination (RI_{germ}), and hybrid phenology ($RI_{phenoHr}$; see Fig. **3a**). *RI* values range from one (complete isolation, implying no interspecific gene flow) through zero (no isolation, implying equal probability of intra- and interspecific gene flow) to minus one (no isolation, implying all gene flow is interspecific; Sobel and Chen, 2014), with *RI* values larger than 0.5 indicating strong barriers (Lowry et al., 2008). Asymmetry values (AS) < 0.15 indicate symmetric barriers, values ≥ 0.15 indicate asymmetric barriers, and values > 0.5 indicate highly asymmetric barriers (Lowry et al., 2008); [1] $RI_{sympField}$ estimated from count data collected in 2016 and 2018 (Fig. **3**) and genotypic information of the local contact site (Fig. **4**); —, not applicable.

Table 2: Pollinator observations in experimental arrays of monospecific and heterospecific plots of *Primula veris* (VE) and *P. vulgaris* (VU) and results of exact binomial tests of goodness-of-fit, testing whether visitation patterns deviated from random expectations for number of (a) plants visited per species, (b) intra- and interspecific pollinator transitions.

	Plot	Species	Transition	Pollinators			
				Large bees	Bee flies	Small bees	Total
(a)	Monospecific	VE	—	22	1	1	24
		VU	—	19	30	39	88
	Heterospecific	VE	—	13	8	0	21
		VU	—	23	33	0	56
	Total	VE	—	35	9	1	45
		VU	—	42	63	39	144
		VE *vs.* VU	—	$P = 0.494$	$\mathbf{P \leq 0.001}$	$\mathbf{P \leq 0.001}$	$\mathbf{P \leq 0.001}$
(b)	Heterospecific	VE	Intraspecific: VE→VE	3	2	—	5
			Interspecific: VU→VE	5	3	—	8
			VE→VE *vs.* VU→VE	$P = 0.727$	$P = 1.000$	—	$P = 0.581$
		VU	Intraspecific: VU→VU	7	14	—	21
			Interspecific: VE→VU	7	2	—	9
			VU→VU *vs.* VE→VU	$P = 1.000$	$\mathbf{P = 0.004}$	—	$P = 0.043$

Pollinators subdivided into large bees (*Anthophora plumipes* and *Bombus* sp.) and bee flies (*Bombylius mayor*) that can both reach nectar at the bottom of the long corolla tubes of *P. veris* and *P. vulgaris*, and small bees that cannot reach nectar at the bottom of the long corolla tubes (*i.e.,* pollen-collecting bees); Arrows indicate direction of transitions; —, not applicable. *P*-values that were significant after strict Bonferroni correction for multiple testing are boldfaced.

Table 3: Strengths and asymmetries of ethological barriers between *Primula veris* and *P. vulgaris* estimated for long-tongued large bees (*Anthophora plumipes* and *Bombus* sp.) and bee flies (*Bombylius major*), short-tongued small pollen-collecting bees, and across all pollinator species for (a) preference (RI_{ethoP}) and (b) constancy (RI_{ethoC}).

		Pollinators			
		Large bees	Bee flies	*Small bees	All pollinators
(a)	Long-styled morph	0.123±0.312	0.725±0.083	0.977±0.023	0.557±0.136
	Short-styled morph	0.123±0.312	0.725±0.083	—	0.395±0.221
	Asymmetry	—	—	—	0.162
(b)	*P. veris*	-0.250	-0.200	—	-0.231
	P. vulgaris	0.000	0.750	—	0.400
	Asymmetry	0.250	0.950	—	0.651

—, not applicable; *small bees transfer pollen only between high organs, thus can only contribute to ethological isolation for L-morphs; for (A) mean values (± standard errors) among the four blocks are presented, for (B) data from all four blocks was pooled. *RI*-values range from one (complete isolation: no interspecific gene flow) through zero (no isolation: equal probability of intra- and interspecific gene flow) to minus one (no isolation: all gene flow is interspecific; Sobel and Chen, 2014), with RI-values larger than 0.5 indicating strong barriers (Lowry et al., 2008). Asymmetry values of < 0.15 indicate symmetric barriers, values ≥ 0.15 indicate asymmetric barriers, and values > 0.5 indicate highly asymmetric barriers (Lowry et al., 2008).

Table 4: Fruit, seed, and seedling formation in intra- *vs.* interspecific treatments from experimental arrays; means and standard deviations for numbers of (a) fruits (proportions of flowers developed into fruits), (b) total seeds (with mean values and ranges from manual crosses by Valentine (1955) and Ernst (1925) added for comparison), (c) filled seeds, and (d) seedlings produced per fruit for long-styled (L-) and short-styled (S-) morphs of *Primula veris* and *P. vulgaris*.

	Data source	Species	Morphs	Treatments	
				Monospecific	Heterospecific
(a)	Exp. arrays	*P. veris*	S	0.840±0.211	0.844±0.210
			L	0.856±0.200	0.809±0.224
		P. vulgaris	S	0.661±0.253	0.635±0.237
			L	0.602±0.163	0.474±0.217
		Treatment	$P = 0.112$		
		Species asymmetries	$P = 0.324$		
		Morph asymmetries	$P = 0.283$		
(b)	Exp. arrays	*P. veris*	S	44.473±17.510	41.055±18.710
			L	41.692±16.762	33.115±19.238
		P. vulgaris	S	36.744±16.709	39.145±17.862
			L	40.304±15.998	27.195±17.293
		Treatment	$P = 0.024$*		
		Species asymmetries	$P = 0.816$		
		Morph asymmetries	$P = 0.003$**	$P = 0.532$	$P = 0.001$***
	Valentine / Ernst	*P. veris*	Both / L	51 (20-69) / 31.4 (—)	46 (17-74) / 20.6 (—)
		P. vulgaris		46 (27-66) / 34.5 (—)	52 (23-79) / — (—)
(c)	Exp. arrays	*P. veris*	S	41.868±17.574	37.264±19.287
			L	39.571±16.571	28.551±19.066
		P. vulgaris	S	33.634±17.888	20.539±18.446
			L	37.076±16.437	18.039±17.773
		Treatment	$P = 0.001$***		
		Species asymmetries	$P = 0.026$*	$P = 0.433$	$P < 0.001$***
		Morph asymmetries	$P = 0.027$*	$P = 0.377$	$P = 0.031$*
(d)	Exp. arrays	*P. veris*	S	10.438±9.280	7.080±8.294
			L	10.000±8.548	4.770±6.915
		P. vulgaris	S	5.103±5.882	2.457±3.479
			L	5.400±5.328	2.547±3.450
		Treatment	$P = 0.019$*		

Species asymmetries	$P = 0.967$
Morph asymmetries	$P = 0.332$

Generalized linear mixed effects (GLMM) and contrast results are presented for intra- *vs.* interspecific (treatment), treatment × species (species asymmetries), and treatment × morph (morph asymmetries) effects. Complete GLMM results are presented in Table **S3**; Sequential Bonferroni correction was implemented to account for multiple tests; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; —, no data.

Figure Legends

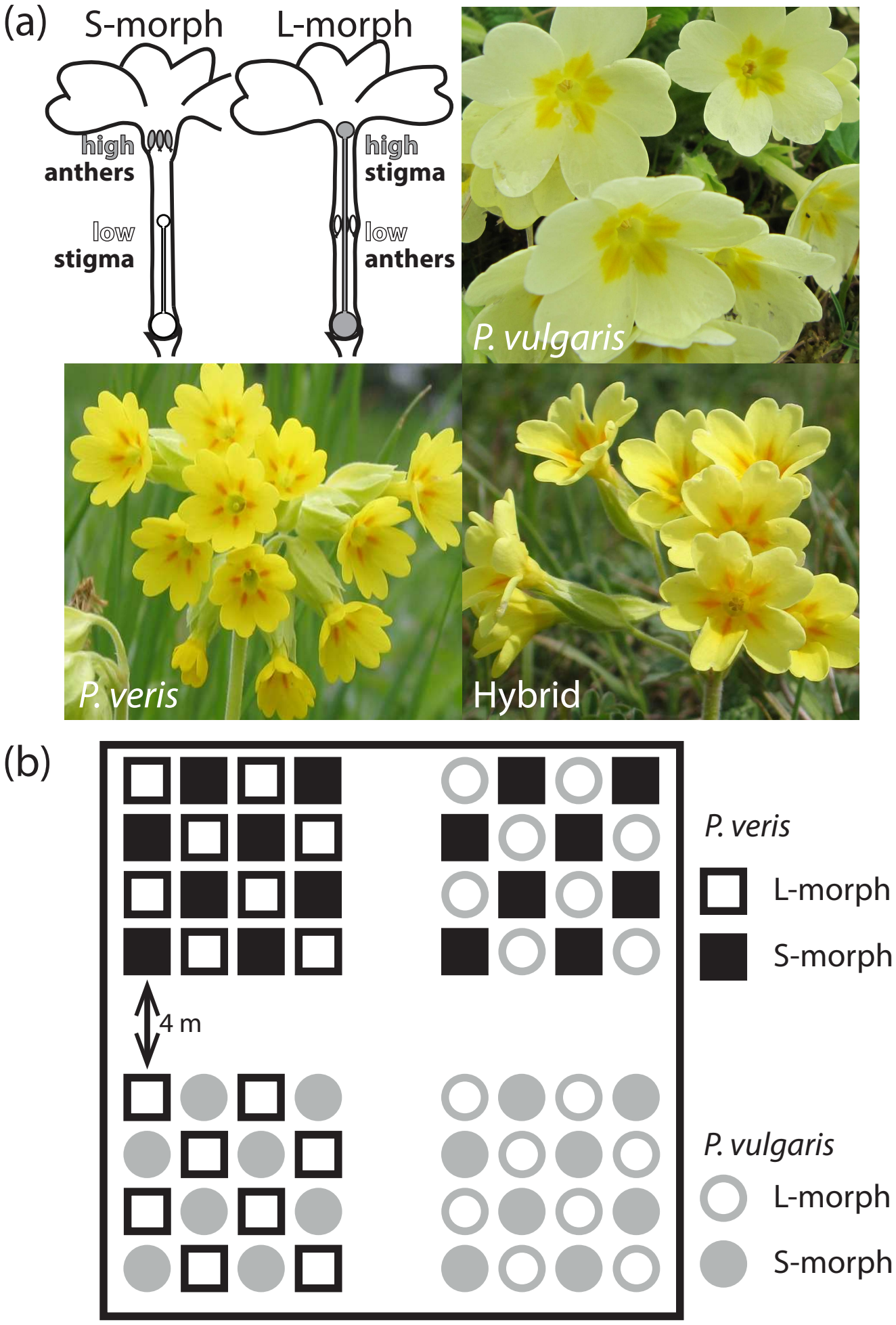
Figure 1: Reciprocal herkogamy in *Primula*, parental and hybrid phenotypes, and experimental design to quantify ethological, seed-developmental, and germination barriers. (a) Diagrams of long-styled (L-) and short-styled (S-) morphs of distylous *Primula ssp.*, with sexual organs placed reciprocally at two levels in the corolla tubes of compatible, heteromorphic flowers (*i.e.*, reciprocal herkogamy; see also Fig. **S1**) and photographs of *P. vulgaris*, *P. veris*, and phenotypically intermediate plant (presumed hybrid). (b) Experimental block containing four plots with 16 potted plants each; two plots (top left and bottom right) represent the monospecific treatments with *P. veris* (squares, black) and *P. vulgaris* (circles, grey), respectively; two plots (bottom left and top right) represent the heterospecific treatments. Each plot contains eight long-styled (L-morph; filled symbols) and eight short-styled (S-morph; empty symbols) plants in alternating order. Panel (**a)** was modified from Keller *et al.*, 2016. Photos were taken by the first author in natural Swiss populations.

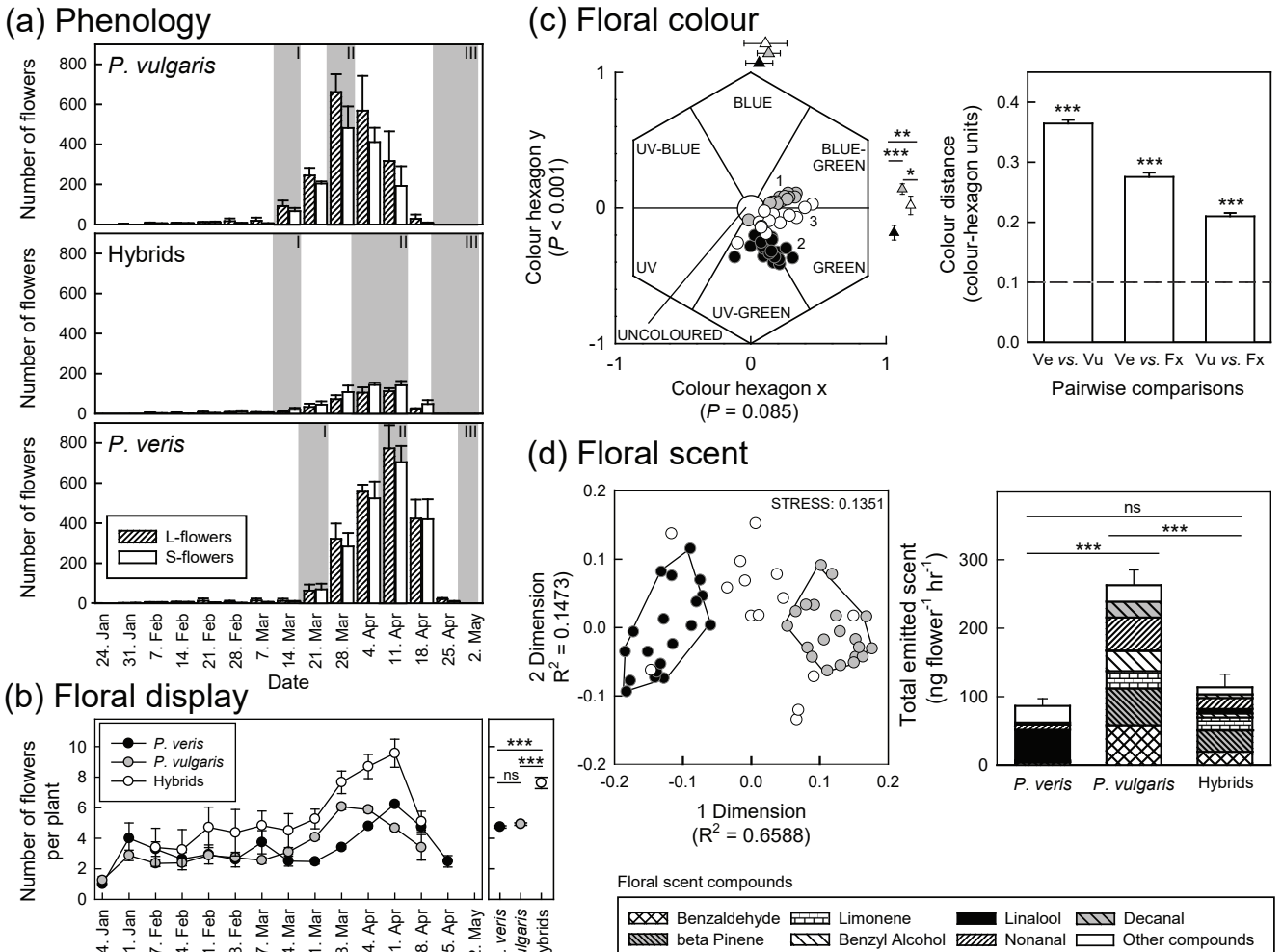
Figure 2: Floral differences between *Primula veris* (Ve)*, P. vulgaris* (Vu) and hybrids (Fx): (a) flowering time, (b) floral display, (c) petal colour, and (d) floral scent. (a) Mean numbers of long-styled (L) and short-styled (S) flowers with standard errors; grey shaded areas mark (I) start of blooming (≥ 2% of all flowers blooming), (II) peak flowering, and (III) end of blooming (all flowers withered). (b) Left panel: Mean number of flowers per plant with standard errors per census day; asterisks indicate dates with only one or two out of the three taxa blooming. Right panel: Mean number of flowers across all dates; significance levels indicate that display sizes were significantly larger in hybrids than parents (*** $P \leq 0.001$) but not significantly different between *P. veris* and *P. vulgaris* $P > 0.05$ (ns). (c) Left panel: Petal colour of individual plants (circles) placed in the hexagon colour space of bees and mean values (triangles) per taxon with standard deviations; significance levels indicate that petal colour differed significantly along the y-axis between *P. veris* and *P. vulgaris*, and between hybrids and either parent (*** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$). Colour for humans: 1 pale-yellow; 2 golden-yellow; 3 lemon (see also Fig. **1a**). Right panel: Means of pairwise colour distances between *P. veris*, *P. vulgaris*, and hybrids with standard errors; the dashed line indicates the minimal colour distance of 0.1 colour-hexagon units for bees to show behavioural colour discrimination (Chittka *et al.*, 2001; Dyer & Chittka, 2004); significance levels indicate that pairwise colour distances were significantly larger (*** $P \leq 0.001$) than 0.1 colour-hexagon units. (d) Left panel: Nonmetric multidimensional scaling (NMDS) plot

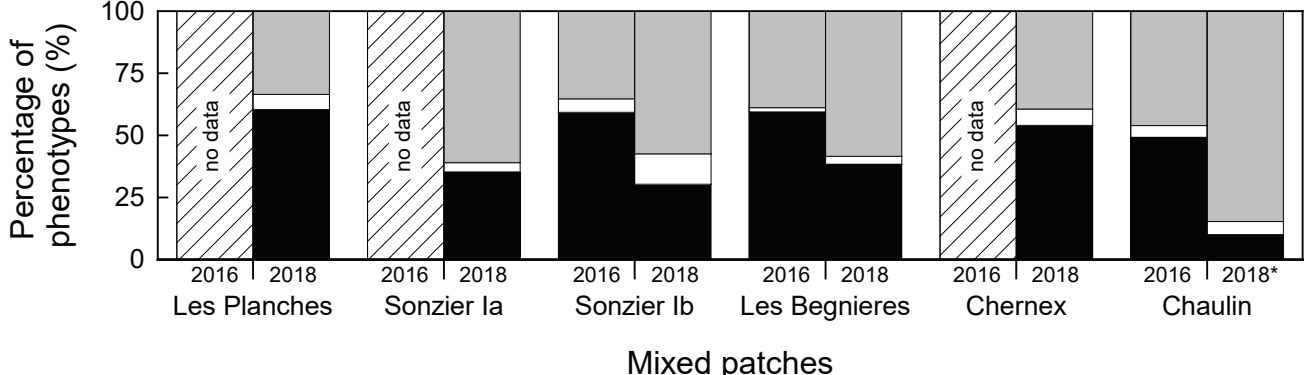
based on 24 floral compounds; the Shepard plot stress value indicates how well the fitted data describe the scent data in the two-dimensional space; stress values < 0.15 represent a good fit. Right panel: Mean emitted scents, standard errors and chemical compositions of *P. veris*, *P. vulgaris*, and hybrids.

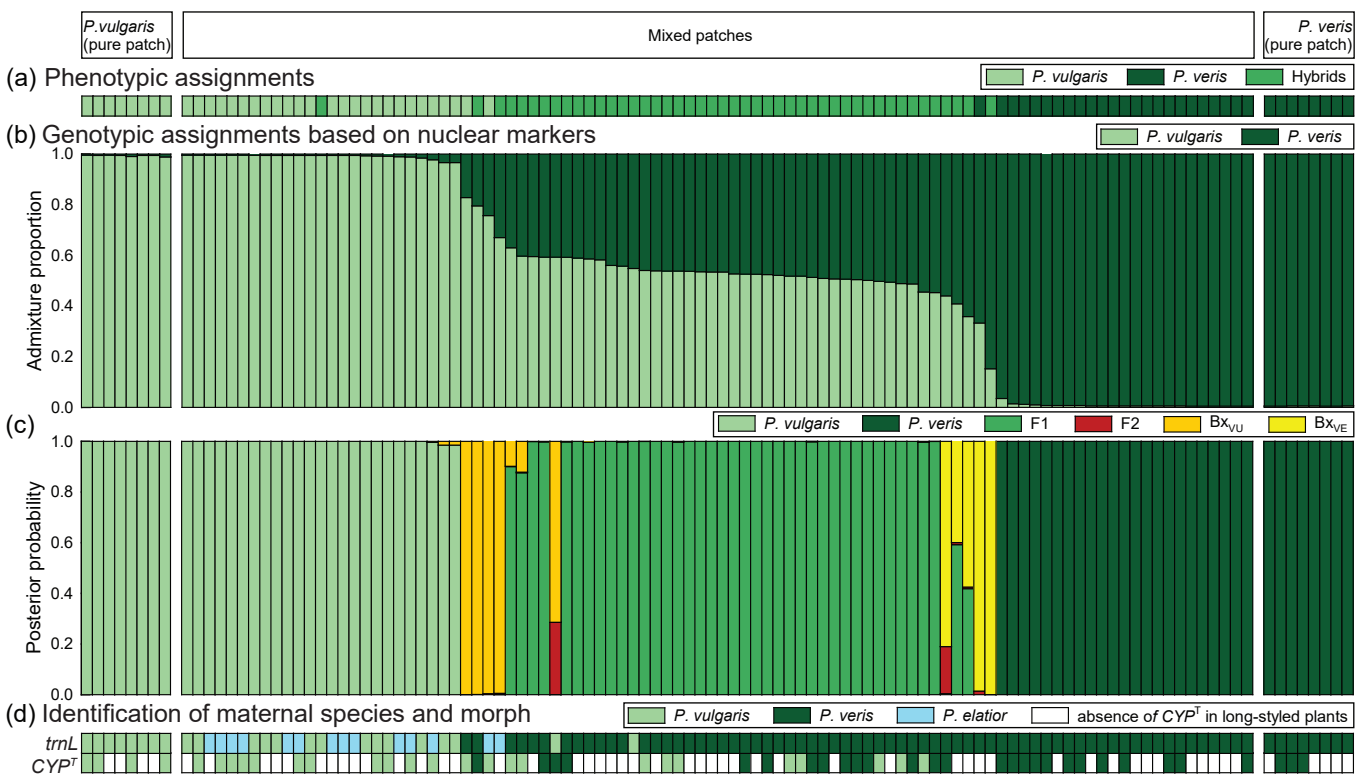
Figure 3: Phenotypic composition of mixed patches in the natural contact site between *Primula veris* and *P. vulgaris* retrieved from population surveys in 2016 and 2018 (see also Table **S1d**). Colour code: black = phenotypic *P. veris*, grey = phenotypic *P. vulgaris*, and white = phenotypic hybrids; Asterisk: data were not used to estimate $RI_{sympField}$ because the phenotypic composition changed dramatically between 2016 and 2018.

Figure 4: Phenotypic and genetic assignments and patterns of hybridization for 112 individuals (eight individuals each of *Primula veris* and *P. vulgaris* from pure patches and 96 individuals from three mixed patches) from a large natural contact site between *P. veris* and *P. vulgaris* in Switzerland. (a) Phenotypic taxa assignments performed in the field. (b) Individual assignment to genetic clusters inferred by STRUCTURE (Pritchard *et al.*, 2000) for $K = 2$ based on nuclear markers. (c) Posterior probability assignments (q) of each individual to *P. veris*, *P. vulgaris*, F1 hybrids, F2 hybrids, and backcrosses to either species (Bx_{VE}, Bx_{VU}) estimated by NewHybrids using Jeffreys-like priors (Anderson & Thompson, 2002) based on nuclear markers. (d) Chloroplast DNA genotypes (upper panel) and heterostyly-supergene genotypes (lower panel) as determined by polymorphisms among *P. veris*, *P. vulgaris*, and *P. elatior* for *trnL* and exon 3 of CYP^T (see Fig. **S6**). In all figures, individuals are ordered by increasing admixture proportion of *P. veris*, as estimated by STRUCTURE. The three replicated analyses in STRUCTURE and NewHybrids gave consistent results with ≤ 0.003 differences for all individuals.


(a)
S-morph
L-morph
high anthers
low stigma
high stigma
low anthers
P. vulgaris
P. veris
Hybrid
(b)
4 m
P. veris
L-morph
S-morph
P. vulgaris
L-morph
S-morph


(a) Phenology
P. vulgaris
Hybrids
P. veris
Number of flowers
I
II
III
L-flowers
S-flowers
24. Jan
31. Jan
7. Feb
14. Feb
21. Feb
28. Feb
7. Mar
14. Mar
21. Mar
28. Mar
4. Apr
11. Apr
18. Apr
25. Apr
2. May
Date
(b) Floral display
Number of flowers per plant
P. veris
P. vulgaris
Hybrids
ns
(c) Floral colour
BLUE
UV-BLUE
BLUE-GREEN
UV
GREEN
UV-GREEN
UNCOLOURED
Colour hexagon y (P < 0.001)
Colour hexagon x (P = 0.085)
Colour distance (colour-hexagon units)
Ve vs. Vu
Ve vs. Fx
Vu vs. Fx
Pairwise comparisons
(d) Floral scent
STRESS: 0.1351
2 Dimension R² = 0.1473
1 Dimension (R² = 0.6588)
Total emitted scent (ng flower⁻¹ hr⁻¹)
Floral scent compounds
Benzaldehyde
beta Pinene
Limonene
Benzyl Alcohol
Linalool
Nonanal
Decanal
Other compounds


Percentage of phenotypes (%)
100
75
50
25
0
no data
no data
no data
2016
2018
2016
2018
2016
2018
2016
2018
2016
2018
2016
2018*
Les Planches
Sonzier Ia
Sonzier Ib
Les Begnieres
Chernex
Chaulin
Mixed patches


P.vulgaris (pure patch)
Mixed patches
P. veris (pure patch)
(a) Phenotypic assignments
P. vulgaris
P. veris
Hybrids
(b) Genotypic assignments based on nuclear markers
P. vulgaris
P. veris
Admixture proportion
1.0
0.8
0.6
0.4
0.2
0.0
(c)
P. vulgaris
P. veris
F1
F2
Bx$_{VU}$
Bx$_{VE}$
Posterior probability
1.0
0.8
0.6
0.4
0.2
0.0
(d) Identification of maternal species and morph
P. vulgaris
P. veris
P. elatior
absence of CYP^T in long-styled plants
trnL
CYP^T